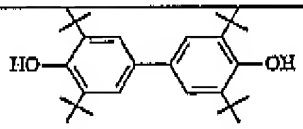
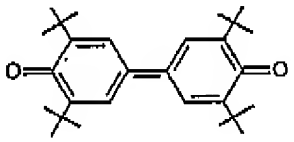
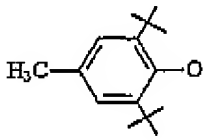
Age matched Control	22.6 (2.4)	5.2 (0.9)	36.6 (3.1)	-
Bisphenol	25.2* (1.2)	6.3 (0.5)	22.9* (0.8)	216 (25)

Animals were fed Lactamin R3-chow containing either 0.03% bisphenol or vehicle alone to act as control. Young and age-matched control animals were sacrificed at 8 and 15 weeks of age, respectively.

Samples of pooled plasma from bisphenol-treated mice were markedly resistant to peroxyl radical-induced ex vivo lipid peroxidation compared with age-matched controls (Fig. 5). Thus, even after 12 h of oxidation at 37 °C, α-TOH remained unaltered (Fig. 5A) with <1 μM CE-OOH detected. By contrast, ~70% of α-TOH was consumed and >30 μM CE-OOH accumulated in the corresponding control plasma (Fig. 5B), fully consistent with plasma lipid peroxidation proceeding via tocopherol-mediate peroxidation (TMP). Separate studies showed that this resistance to peroxyl radial-induced *ex vivo* lipid peroxidation was directly attributable to bisphenol, as the bisphenol rather than α-TOH was consumed during the period of oxidation. The corresponding oxidation product, diphenoquinone, is incapable of acting as an co-antioxidant, as judged by its high anti-TMP index and inability to cause the decay of α-tocopheroxyl radical (Table 8).

Table 8. Anti-TMP and tocopheroxyl radical attenuating ability (TRAA) indices for bisphenol, diphenoquinone and BHT.

Compound	Structure	Anti-TMP index*	TRAA‡
H 212/43		3.2	Immediate decay
H 330/68		100	$k_{obs(+)}/k_{obs(-)} \sim 1$
BHT		8-10	Immediate decay

Pharmacological Inhibition of ABCA1 Biodegradation Increases HDL Biogenesis and Exhibits Antiatherogenesis

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Objective -- Expression of ATP binding cassette transporter (ABC) A1 is regulated by transcription of the gene and by calpain-mediated proteolytic degradation, and inhibition of the latter reaction results in increase of ABCA1 and HDL biogenesis in vitro. We examined whether this approach could be a target for antiatherogenic treatment.

Methods and Results -- Spiroquinone and diphenoquinone, oxidized products of probucol, inhibited ABCA1 degradation without inhibiting its activity and without changing transcription of the gene, and accordingly increased apoA-I/ABCA1-dependent generation of HDL in vitro. Both compounds increased hepatic ABCA1 and plasma HDL when given to rabbits without inceasing antioxidative activity in plasma. Both increased plasma HDL and decreased vascular lipid deposition in cholesterol-fed rabbits.

Conclusion -- Inhibition of calpain-mediated degradation of ABCA1 by spiroquinone or diphenoquinone increased HDL biogenesis in vitro and in vivo, and prevented atherogenesis in hypercholesterolemic rabbits, indicating that this is a good drug model for raising HDL and preventing atherogenesis.

Key Words: ABCA1, HDL, Atherosclerosis, calpain, probucol,

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Short Summary.

Spiroqunone and diphenoquinone, oxidized products of probucol, inhibited calpain-mediated degradation of ABCA1 without inhibiting its activity. This reaction increased HDL biogenesis in vitro and in vivo, and prevented atherogenesis in hypercholesterolemic rabbits, indicating that this is a good drug model for raising HDL and preventing atherogenesis.

Running title: Increase of HDL by inhibition of ABCA1 Biodegradation

HDL plays a central role in the cholesterol catabolic pathway by transporting it from extrahepatic cells to the liver for conversion to bile acids, and accordingly is thought to be antiatherogenic. Apolipoprotein-mediated generation of new HDL from cellular lipid is one of the major events in the initial step of this pathway for cell cholesterol release[1, 2]. This reaction was found defective in genetic HDL deficiency, Tangier disease[3, 4], and mutations were identified in the gene of ATP-binding cassette transporter A1 (ABCA1) to cause this disorder[5-7].

Activity of ABCA1 is regulated both by the gene expression and calpain-mediated proteolysys. ABCA1 is stabilized against this biodegradation by helical apolipoprotein[8-10] and destabilized by unsaturated fatty acid[11] or by overloaded cholesterol[12]. As inhibition of calpain increases HDL biogenesis in cultured cells[8], proteolytic degradation of ABCA1 is a potential drug target for increasing HDL. ABCA1 degradation takes place intracellularly, and its inhibition recycles ABCA1 to the cell surface[13]. Direct inhibition of ABCA1 internalization also causes its accumulation in the cell surface[13]. In both cases HDL biogenesis increases, indicating it takes place in the surface[13]. Therefore, inhibition of any of these steps would cause suppression of ABCA1 biodegradation and would be a candidate target for increase of HDL biogenesis and preventing or regressing atherosclerosis. However, it has not been demonstrated that such approach is actually feasible by showing pharmacological inhibition of ABCA1 biodegradation results in increase of plasma HDL and prevention of atherogenesis with good candidate seed compounds.

A hypolipidemic drug probucol is known to reduce plasam HDL[14] by inhibiting ABCA1-mediated HDL biogenesis[15] to exhibit a similar phenotype to Tangier disease[16]. Interestingly, probucol causes not only inactivation of ABCA1 but also inhibition of its biodegradation[17]. We found in our preliminary experiments that the crude oxidized products of probucol enhanced HDL biogenesis rather than inhibited it in cultured cells. Based on these findings, we hypothesized that some of the compounds in this mixture may function as inhibitors of ABCA1 degradation without inhibiting its activity. We therefore investigated functions of spiroquinone (SQ) and diphenoquinone (DQ) (Supplementary Figure I)[18], whether they inhibit ABCA1 biodegradation without influencing its activity and increase HDL biogenesis in vitro and in vivo.

Materials and Methods

Cell lines and Culture Conditions.

THP-1 cells were maintained in 10 % fetal bovine serum-RPMI1640 (Sigma) in a humidified atmosphere of 5 % CO_2 and 95 % air at 37°C. Human monocytic cell line cells THP-1 were differentiated to macrophages (THP-1 macrophages) by culturing the cells at a density of 1.0 x 10^6 cells/ml in the presence of 3.2 x 10^{-7} M of phorbol 12-myristate 13-acetate (Wako) for 24 hrs[8, 10]. Balb/3T3 fibroblasts and HEK293 cells were maintained in 10 % fetal bovine serum-DMEM (Sigma). The cells were seeded in culture plates at a density of 3 x 10^5 cells/ml and cultured for 3 days before use.

Loading the Compounds to the Cells.

Probucol was obtained from a commercial source and its oxidative products SQ and DQ were synthesized by an in vitro oxidation of probucol and isolated[18]. Probucol, SQ or DQ was given to the cells either being incorporated into acetyl LDL as a vehicle[15, 17] or directly as a solution in 2-butanol. Drug-containing acetyl LDLs were prepared as described previously in detail[15]. Briefly, human LDL was incubated with sonicated lipid microemulsion composed of egg phosphatidylcholine (Avanti), triolein (Wako) and a selected compound in the presence lipoprotein-free human plasma fraction, re-isolated by a dextran sulfate-cellulose column and ultracentrifugation, and acetylated with acetic anhydride. The final preparation contained approximately 0.3 μg of the respective compound per 100 μg protein. THP-1 macrophages were preloaded with respective compound by incubating with acetyl LDL containing it for 24 hrs. The compounds were alternatively given to the cells by directly adding them as a solution in 2-butanol, to make the final solvent concentration 0.5 % in the culture medium.

Cellular Lipid Release by apolipoprotein A-I (apoA-I).

ApoA-I was isolated from human HDL fraction as described previously[19]. THP-1 macrophages were preloaded with probucol, SQ or DQ by incubating the compound-containing acetyl LDL and incubated in the media containing 0.2 % bovine serum albumin (BSA) (Sigma) and 10 μg/ml of apoA-I for 24 hrs. The compounds were also given as a 2-butanol solution as mentioned above, by incubating the cells in the presence of the compounds and apoA-I for 24 hrs. Cholesterol and choline-phospholipid released into the media were measured enzymatically (Wako)[20]. The cells were dissolved in 0.1N NaOH for protein determination by BCA method (Pierce).

Western Blotting.

After the cells were incubated to load the compounds, they were suspended in 5 mM Tris-HCl buffer (pH 8.5) containing protease inhibitor cocktail (Sigma) and placed on ice for 30 min. The cell suspension was centrifuged at 650 x g for 5 min, and the supernatant was centrifuged at 105,000 x g for 30 min to precipitate total membrane fraction. The liver specimens, 20 μg wet weight, of the rabbits were treated in the same manner as preparation of the membrane fraction. Protein in these fractions was analyzed by Western blotting using specific polyclonal antibodies against ABCA1[8], scavenger receptor B1 (Novus Biologicals) and β-actin (Sigma), and visualized by a chemiluminescence method (Amersham life science).

Real time Quantitative Polymerase Chain Reaction (PCR).

The expression of ABCA1 was measured by using the probes previously reported for human and mouse[8] in a 7300 Real Time PCR System (Applied biosystems). Cultured cells were lysed in the presence of phenol and guanidine thiocyanate. cDNAs were synthesized by SuperScript™ First-Strand Synthesis Systems (Invitrogen). For rabbit ABCA1, total RNA was purified from the rabbit liver and cDNA was synthesized as described above. A partial sequence of ABCA1 was amplified with synthetic oligonucleotide primers (5'-ACA ATA GTT GTA CGA ATA GCA GGG-3', 5'-CTC ATC CTG TAG AAA AGA TGT GAG-3') and cloned into pGEM□-T Easy Vector (Promega). As the sequence of the partial clone of rabbit ABCA1 analyzed by a capillary sequencer 3100 (ABI) was 97 % homologous to human ABCA1, these primers were used for the PCR. The expression was standardized for glyceraldehydes-3-phosphate dehydrogenase and β-actin.

Metabolic Analysis of ABCA1.

To examine biodegradation of ABCA1, THP-1 macrophages or Balb/3T3 cells were incubated for 24 hrs with 9-*cis*-RA (Sigma) to increase the expression of ABCA1, and treated with SQ or DQ for 30 min in 0.2 % BSA-RPMI1640. Cells were washed once with PBS and incubated in 0.2 % BSA-RPMI1640 containing 140 μM of cycloheximide (Sigma) for the indicated periods. Expression of ABCA1 was analyzed by Western blotting. ABCA1 in cell surface was analyzed by biotinylation of the surface protein and its precipitation with abidin-beads followed by its Western blotting[13]. Internalization of ABCA1 was analyzed by biotinylation and its cleavage of surface ABCA1 as described elsewhere[13]. To visualize intracellular localization of ABCA1, expression vector that contains ABCA1-green fluorescent protein (GFP) hybrid cDNA was transfected and

expressed in HEK293 cells as described previously[21]. Expression of ABCA1-GFP protein was confirmed by Western blotting with anti-ABCA1 antibody and with anti-GFP antibody. Intracellular localization of ABCA1-GFP was visually demonstrated fluorescent image of the living cells, placed onto a 50-mm round coverslip for mounting in a temperature-controlled chamber at 37°C, were viewed with a LSM510 PASCAL laser scanning confocal microscope (Carl Zeiss). The averaged fluorescent intensity of ABCA1 in plasma membrane was measured for randomly selected 60 cells by using the software of the LSM510 PASCAL microscope.

Animal Experiments.

Male New Zealand White rabbits (3-month old) were fed with LRC-4 diet containing SQ and DQ for 7 days. Plasma lipoproteins were analyzed for HDL and non-HDL fractions separated by ultracentrifugation at densities above and below 1.063 g/mL. Purity of each fraction was verified by agarose electrophoresis, and its cholesterol was measured by the enzymatic method. Expression of ABCA1 in the liver was determined by quantitative PCR for mRNA and by Western blotting for protein as described above. For high-cholesterol experiments, male New Zealand White rabbits (3-month old) were fed with 0.5 % cholesterol-containing diet supplemented with SQ or DQ for 8 weeks. Plasma lipoproteins were measured as described above. Aorta was extracted and fixed with 10 % neutral buffered formalin solution, and lipid deposition on the lumen surface was stained with Oil Red O. The anti-atherogenic effect of the drugs was evaluated by measuring the Oil Red O-stained area in the thoracic and abdominal regions. The digitalized images were analyzed by using softwares of Adobe Photoshop and NIH Image to estimate the relative area of lipid-deposit.

Antioxidative Activity in Plasma

To measure antioxidaive activity of the compounds in vivo, WHHL rabbits (3 month old) were fed LRC4 diet containing SQ or probucol for 1 month. The serum, 5 □l, was used for estimation of the anti-oxidant activity by evaluating its activity to reduce Cu^{++} to Cu^{+}, as measuring 490 nm of a stable complex of Cu^{+}/bathocuproine[22], based upon the principle by MEDICAL, Italy, and modified by Japan Institute for The Control of Aging, Nikken SEIL Corporation, according to the manufacture's instruction.

Cholesteryl Ester Transfer Protein (CETP) Mass in Plasma

CETP mass in rabbit plasma was measured by enzyme-linked immuno-sorbent assay as described previously[23] by using an assay system provided from Sekisui Medical Co. Ltd

(Tokyo).

Other Methods

Intensity of each band was digitally scanned and semi-quantified by using an EPSON GT-X700 and Adobe Photoshop software. Statistical analysis of the data was performed by 1-way ANOVA followed by Scheffé's test. Values represent average ± SD for at least three independent data.

Results

Probucol, SQ and DQ were incorporated into acetylated LDL and fed to THP-1 macrophages, and cellular lipid release by apoA-I was measured. While release of cholesterol and phospholipid was inhibited by probucol[15, 17] (Figure 1A), SQ and DQ enhanced the lipid release (Figure 1A). ABCA1 protein was markedly increased by probucol in spite of inhibition of the HDL biogenesis, being consistent with our previous finding[17] (Figure 1B). SQ and DQ also markedly increased ABCA1. Other downstream oxidative products of probucol[18], bisphenol and butylphenol, did so but to a less extent (data not shown). When the THP-1 macrophages preloaded with acetylated LDL was incubated with SQ and DQ added in a 2-butanol solution, ABCA1 apparent increased in the initial 30 min of the incubation (Figure 1C). The similar effects are shown in a dose-dependent manner of SQ and DQ when the compounds were given to the cells in a 2-butanol solution in THP-1 macrophages (Supplementary Figure IIA) and Balb/3T3 mouse fibroblasts (Supplementary Figure IIB). However, the message of ABCA1 was not influenced by either compound (Supplementary Figure IIC), similar to the previous finding with probucol[17].

Figure 2A and 2B show the decay of ABCA1 in the presence of cycloheximide. Both SQ and DQ apparently retarded this process. Figure 2C demonstrates inhibition of ABCA1 internalization by these compounds. Internalization of surface ABCA1 pre-labeled with biotinylation was inhibited by SQ and DQ shown in the left panel. In contrast, ABCA1 in the cell surface increased by these compounds, shown in the right panel. Inhibition of ABCA1 biodegradation by these compounds was thus shown to be by inhibiting internalization of ABCA1[13]. Effect of SQ and DQ on intracellular localization of ABCA1 was further examined by using HEK293 cells in which ABCA1-GFP was over-expressed. Figure 3A shows increase of transfected

ABCA1-GFP by SQ and DQ. Figure 3B shows increase of fluorescence intensity of ABCA1-GFP as well as images of its intracellular localization. SQ and DQ increased the fluorescence intensity along with cellular surface. In these conditions, SQ and DQ increased the release of cellular lipid by apoA-I (Figure 3C).

SQ and DQ were given to rabbits to examine their in vivo effects. Figure 4A shows increase of plasma HDL-cholesterol and of hepatic ABCA1 protein (also in Supplementary Figure III) by SQ and DQ, while no increase of ABCA1 mRNA. Since probucol has strong anti-oxidative activity and its beneficial effects of antiatherogenesis are assumed to be due to this function, anti-oxidative activity of SQ, that supposedly has higher anti-oxidative activity than other probucol oxidative products, was examined in vivo in comparison to probucol. While probucol caused substantial anti-oxidative activity in plasma, no significant change was found with higher dose of SQ in plasma anti-oxidative activity (Figure 4A). Figure 4B shows the increase of HDL-cholesterol in the cholesterol-fed rabbits, as shown in a time course, and as its integrated values for the entire experimental term of 8 weeks. SQ and DQ induced the significant increase of HDL, while neither compound caused significant change in non-HDL lipoprotein-cholesterol. The effect of increase of HDL seemed somewhat diminished after the 4-week treatment. Plasma CETP markedly increased as the rabbits being fed with cholesterol without showing difference among the treatment group (Supplementary Figure IV). Increase of ABCA1 protein in the liver by SQ or DQ was retained at the end of the experiment while SR-B1 protein showed no change (Supplementary Figure IV). There was no apparent adverse effect in the animals including change in the body weight.

Figure 5 shows the effects of SQ and DQ on the vascular lesions in the cholesterol-fed rabbits characterized as above. Lipid deposit on the aortic surface was examined by Oil red O staining. Relative lipid deposit area was 0.46 ± 0.19 for the controls versus 0.27 ± 0.09 and 0.29 ± 0.13 for the SQ and DQ treatment groups ($p = 0.02$ and 0.03 against the control), respectively, including the aortic arch regions (Supplementary Figure V). The evaluation for the arch regions, however, may be inaccurate and unreliable as the wall can not be set flat for photographs, so that further quantitative analysis was performed for the thoracic and abdominal regions of aorta (Figure 5A). The lesion area was significantly decreased by SQ (Figure 5B left). When the lesion area was standardized for the integrated value of non-HDL cholesterol in an

individual animal, reduction of lipid deposit was significant both by SQ and DQ (Figure 5B middle). The lipid deposit was a function of (non-HDL cholesterol)/(HDL cholesterol) being regressed to lines with similar parameters for SQ, DQ and total (Figure 5B right), so that the effect of SQ and DQ on the lipid deposition should be attributed to the increase of HDL in association with stabilization of ABCA1.

Discussion

In order to find whether inhibition of ABCA1 biodegradation increases HDL biogenesis and plasma HDL level, we attempted to screen potential candidate chemicals that inhibit proteolytic degradation of ABCA1, including oxidized products of an ABCA1 inactivator, probucol such as SQ and DQ[18] as its crude oxidized products increased HDL biogenesis rather than decreased it in our preliminary experiments. SQ and DQ were found to increase ABCA1 protein and apoA-I-mediated HDL biogenesis in vitro. Both compounds stabilized ABCA1 against calpain-mediated degradation without changing its transcription. They also increased ABCA1-GFP transfected and overexpressed with a non-physiological promoter in HEK293 cells. The compounds increased plasma HDL in rabbits by increasing hepatic ABCA1 and suppressed the lipid deposition in arterial walls in the cholesterol-fed rabbits. Thus, these compounds increase HDL biogenesis through protecting ABCA1 from degradation and prevent atherogenesis in the experimental animals. The effects were apparently independent of antioxidative activity that was considered as one of the major antiatherogenic natures of probucol in similar animal models[24, 25], since these compounds did not exhibit significant antioxidative activity in plasma.

We thus demonstrated that pharmacological inhibition of ABCA1 biodegradation could increase ABCA1 and plasma HDL, and counteract atherogenesis in hypercholesterolemia in vivo. SQ and DQ were shown to inhibit ABCA1 biodegradation seemingly by inhibiting internalization of ABCA1 that is prerequisite for its calpain-mediated proteolysis[13], rather than by direct inhibition of calpain reaction. At this stage, we do not have data to discuss further mechanistic insight for the action of SQ and DQ but the effects might apparently be similar to the effect of cytochalasin D observed in vitro in the cultured cells[13]. Since both SQ and DQ are extremely hydrophobic and likely incorporated into the membrane, these compounds may induce conformational alteration of ABCA1 to stabilize it against internalization for its

degradation. It is interesting that probucol inactivates ABCA1 for both HDL biogenesis and its biodegradation but SQ and DQ only interfere with the latter reaction. There may be a hint in this discrepancy to solve the question on the reaction mechanism of these compounds.

The results demonstrated here showed a novel concept for drug development, to enhance the function of a specific membrane protein such as transporters or receptors by inhibiting their biological degradation. SQ, DQ or their related compounds can thus be potential drug candidates for increase of HDL biogenesis and prevention/cure of atherosclerosis by inhibiting ABCA1 biodegradation. Several issues remain to be addressed. The compounds are extremely hydrophobic and need to be improved for oral administration. Apparent tendency of diminishing the HDL-raising effect may be related such a problem and mechanism for this should be investigated including monitoring ABCA1 expression for a long-term administration. Although probucol has been used in the market for years and these compounds may be produced as its metabolites in the body, it is still important to examine what this type of chemicals cause in animal bodies. Further investigation is required for this type of compound to influence metabolism of membrane proteins in detail in general, including an exact mechanism for inhibiting biodegradation of ABCA1. Wide and through survey is needed for their influence on expression of genes.

Probucol decreases HDL by inhibiting the activity of ABCA1[15-17, 26, 27]. In spite of this HDL-lowering effect, however, probucol was proposed to have specific antiatherogenic nature, being based on clinical findings of efficient regression of cutaneous and tendinous xanthomas in familial hypercholesterolemia[28] and on the prevention of atherogenesis in the experimental animals in association with its antioxidative nature to prevent atherogenic modification of LDL[24, 25, 29]. We previously discovered that probucol inactivates ABCA1 for its biological activity of HDL biogenesis and for its calpain-mediated degradation[17], which likely causes severe reduction of HDL. In contrast, we here demonstrated that the oxidized products of probucol retained the inhibitory effect only on ABCA1 biodegradation but not for HDL biogenesis. If SQ and/or DQ are produced during the in vivo oxidative metabolism of probucol, these products may induce the increase of active ABCA1 in some specific tissues. In addition to the effect on ABCA1, probucol has been proposed to induce apparent increase of the activity of plasma CETP[30] or SR-B1[31] as the causes of the HDL

decrease while we found no change in SR-B1 protein by SQ or DQ in the rabbit liver (Supplementary Figure IV) and in the mRNAs of apoA-I, LCAT, PLTP, SR-B1 in the liver of the probcol-fed mice[16]. Since the HDL-increasing effects of SQ and DQ were observed in mice as well in our preliminary experiments, we at least assume that the effect of SQ and DQ are not related to CETP. On the other hand, CETP markedly increased in the rabbit plasma regardless of the drug administration presumably by cholesterol feeding[32] (Supplementary Figure IV), and this might cause apparent canceling of the specific increase of plasma HDL by SQ and DQ.

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Disclosures

Aska Pharmaceutical Company has no financial and intellectual commitment to this work. Shinji Yokoyama has been involved in establishment of a venture industry Hykes Laboratories. Otherwise the authors declare no conflict of interest.

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Figure Legends

Figure 1. The effect of probucol, spiroquinone (SQ) or diphenoquinone (DQ) in THP-1 macrophages. **A.** Cellular lipid release by apoA-I. The cells were incubated with acetylated LDL containing each compound for 24 h, at 100 μg/mL protein as 1.9 μM probucol, 2.0 μM SQ and 2.4 μM DQ, and incubated with apoA-I, 10 μg/mL, for another 24 h. Cholesterol (CH) and phospholipid (PL) in the medium were determined. **B.** ABCA1 protein in the same condition. Controls (C1 and C2) represent absence and presence of control acetyl LDL. The numbers represent band intensity relative to C2. **C.** Time-dependent increase of ABCA1 after SQ and DQ were added as a 2-butanol solution (25 nM and 0.05 nM, respectively). The graph represents band intensity relative to time zero. The data represent the mean ± SE for 3 samples. * $p < 0.05$, ** $p < 0.01$ in comparison to control (A), to C2 (B), and to time zero (C).

Figure 2. Stabilization of ABCA1 by SQ and DQ. **A.** Retarded degradation of ABCA1 in the presence of cycloheximide in THP-1 macrophages and Balb/3T3 cells, by SQ (25 nM for THP-1 and 20 nM for Balb/3T3) and DQ (0,05 nM and 1.0 nM). **B.** Graphic expression of the results typically represented by Figure 2A after standardization for β-actin. Error bars indicate SE for three measurements. Significant difference from control at each time point is indicated as * $p < 0.05$ and ** $p < 0.01$. **C.** Internalization of ABCA1. Left panel: THP-1 cells were pre-incubated with SQ (25nM) and DQ (0.25 nM) for 16 h to equilibrate the cells with the compounds. The surface ABCA1 were then labeled by biotinylatin and the cells were incubated for time indicated. The surface biotinylation was cleaved and the remaining biotinylated ABCA1 was analyzed as the protein internalized. Right panel: Cell surface ABCA1 was analyzed by surface biotinylation after incubation with SQ and DQ (as indicated in nM) for 1h.

Figure 3. Intracellular localization of ABCA1-GFP in HEK293 cells. **A.** HEK293 cells with stable expression of ABCA1-GFP were cultured with each compound (SQ 50 nM, DQ 0.5 nM) for 12 h. Cellular ABCA1-GFP was analyzed by using anti-ABCA1 antibody. **B.** Fluorescent image of the living cells was viewed with a laser scanning confocal microscope. The averaged fluorescent intensity in plasma membrane was measured by using the software of the LSM5 Pascal microscope. 60 cells were analyzed

in each group. (low magnitude and high magnitude). **C.** Release of cholesterol (CH) and phospholipid (PL) by apoA-I, 10 μg/ml, during the 12-hour incubation. The data represent the mean ± SE for 3 measurements. * $p < 0.05$, ** $p < 0.01$ in comparison to each control.

Figure. 4. Effects of SQ and DQ on rabbit plasma lipoproteins. **A.** The compounds were orally given (mg/kg/day, n = 4) and HDL-cholesterol was measured at the day 3. Hepatic ABCA1 protein and mRNA were analyzed at the day 7. Plasma antioxidative activity was determined for the animals given probucol, 330 mg/kg/day, and SQ, 125 and 250 mg/kg/day, for 1 month. DQ was given in a higher relative dose to SQ than in the in vitro studies due to less solubility in oil indicating poor absorption. **B.** Long term effects of SQ and DQ on plasma HDL-cholesterol. SQ or DQ, 25 mg/kg/day, was given to the animals fed with 0.5 % cholesterol diet for 8 weeks (n=8 in each group). The left panel, plasma HDL-cholesterol. The middle panel, integrated HDL-cholesterol as sum of HDL-cholesterol for the test period (day 3 and at every week thereafter). The right panel, integrated non-HDL-cholesterol estimated similarly to HDL. The data represent the mean ± SE. * $p < 0.05$, ** $p < 0.01$ in comparison to each control.

Figure. 5. Effects of SQ and DQ on vascular lipid deposit in cholesterol-fed rabbits. After 8 weeks of the experiments described in Figure 4, lipid deposit in the aortic wall was evaluated by Oil Red-O staining. **A.** Lipid deposit in the thoracic and abdominal aorta. **B.** Digitalized images were analyzed by an image processing software. Left panel, the lesion relative area (%). Middle panel, the relative lesion area standardized by the integrated non-HDL-cholesterol in each animal. Right panel, plot of the relative lesion area (%) against the index of (integrated non-HDL-cholesterol)/(integrated HDL-cholesterol). Solid line represents fitting for all the groups ($y = 0.46 x + 11.2$, $r = 0.528$); Even-broken line for the SQ-fed group ($y = 0.51 x + 3.3$, $r = 0.81$); and uneven-broken line for the DQ group ($y = 0.59 x + 5.2$, $r = 0.83$).

Figure 1, Arakawa et al.

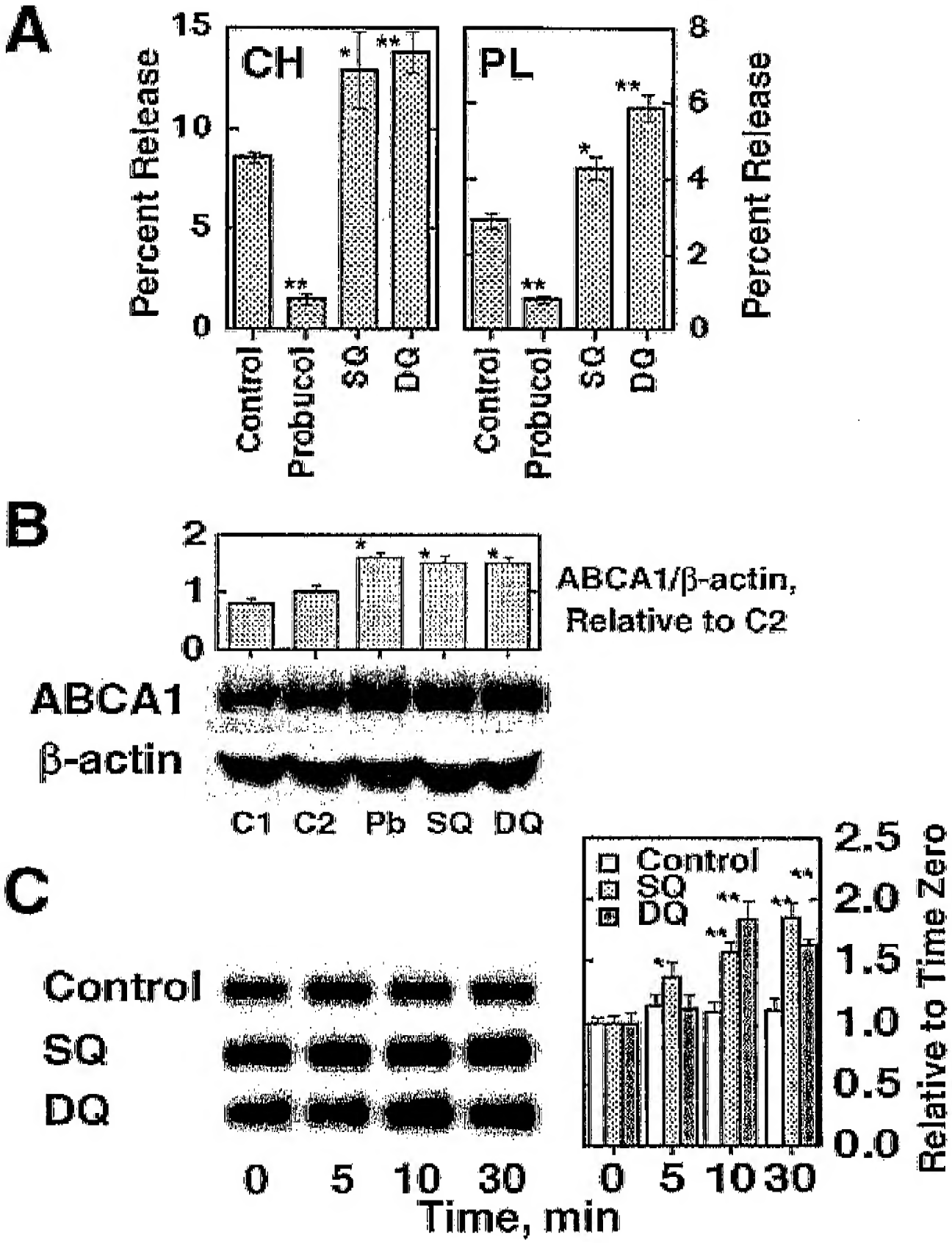


Figure 2, Arakawa et al.

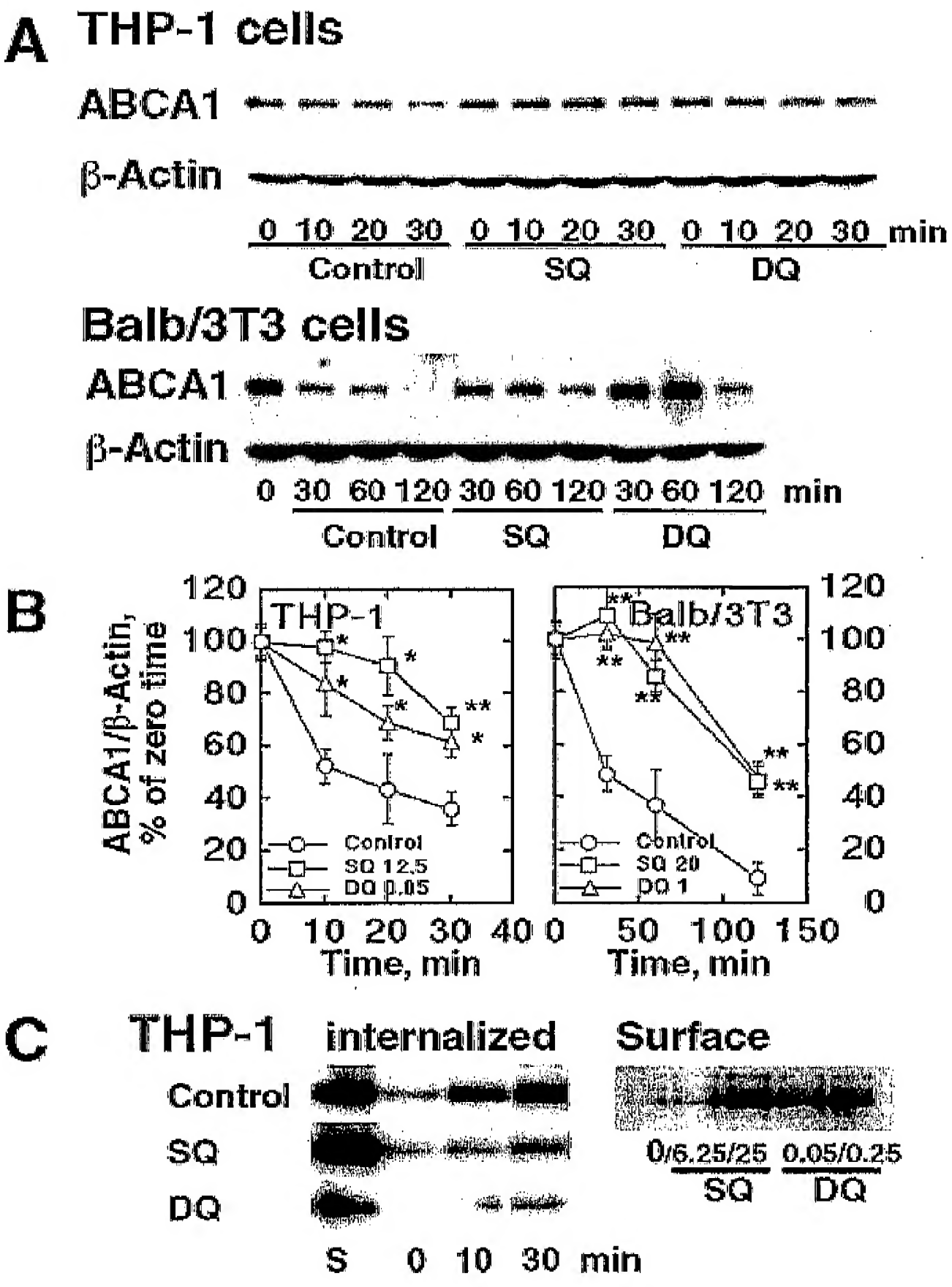


Figure 3, Arakawa et al.

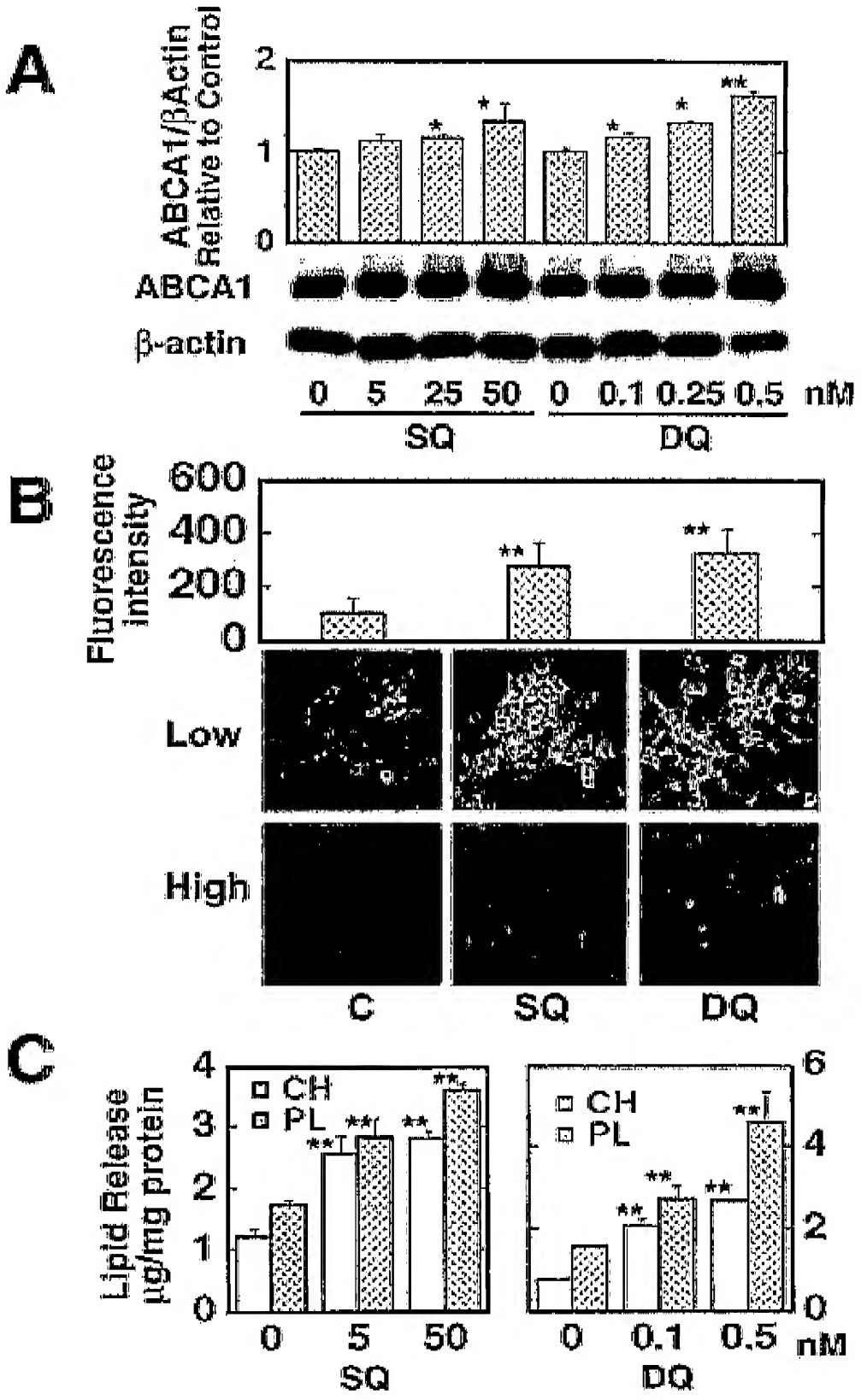


Figure 4, Arakawa et al.

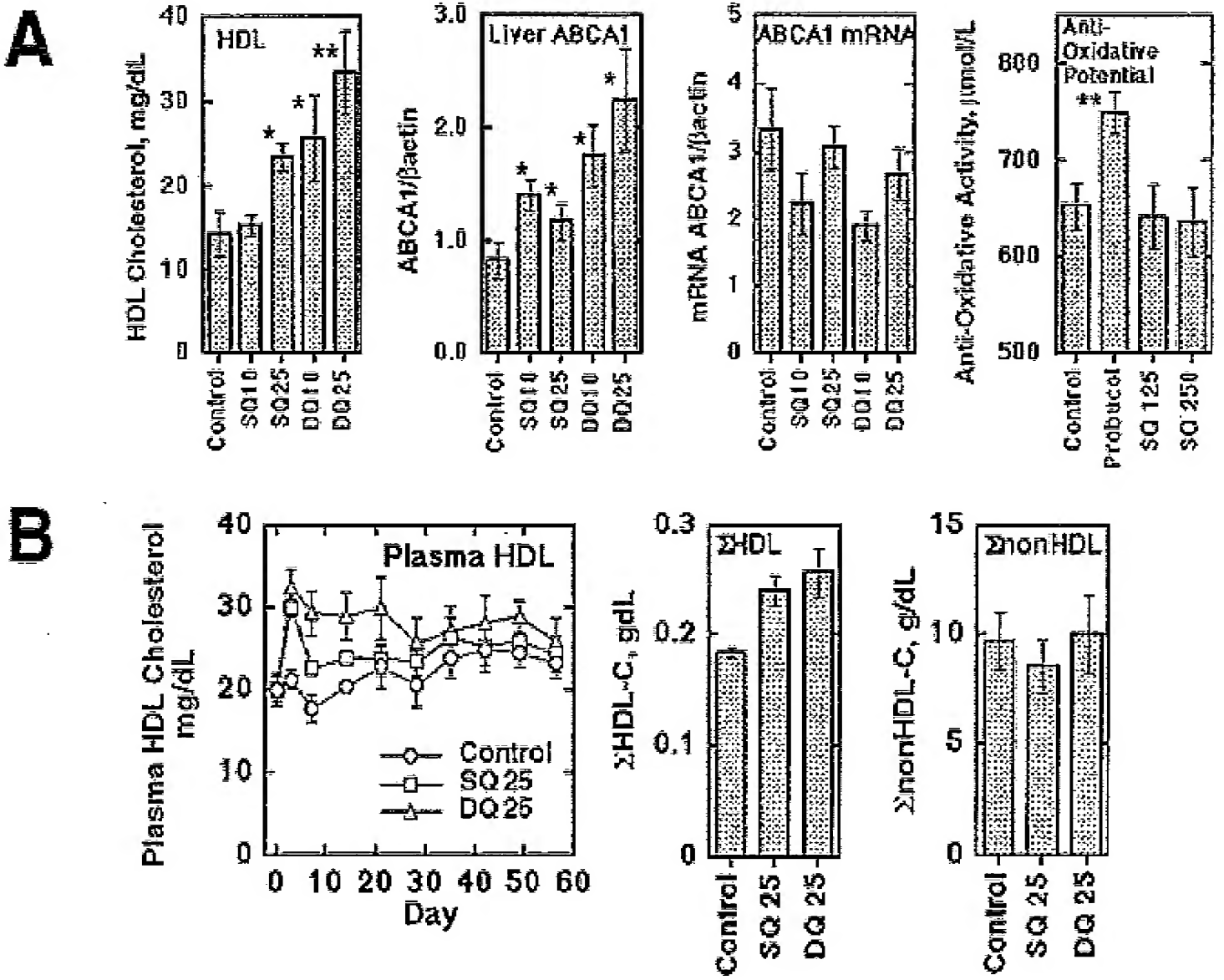
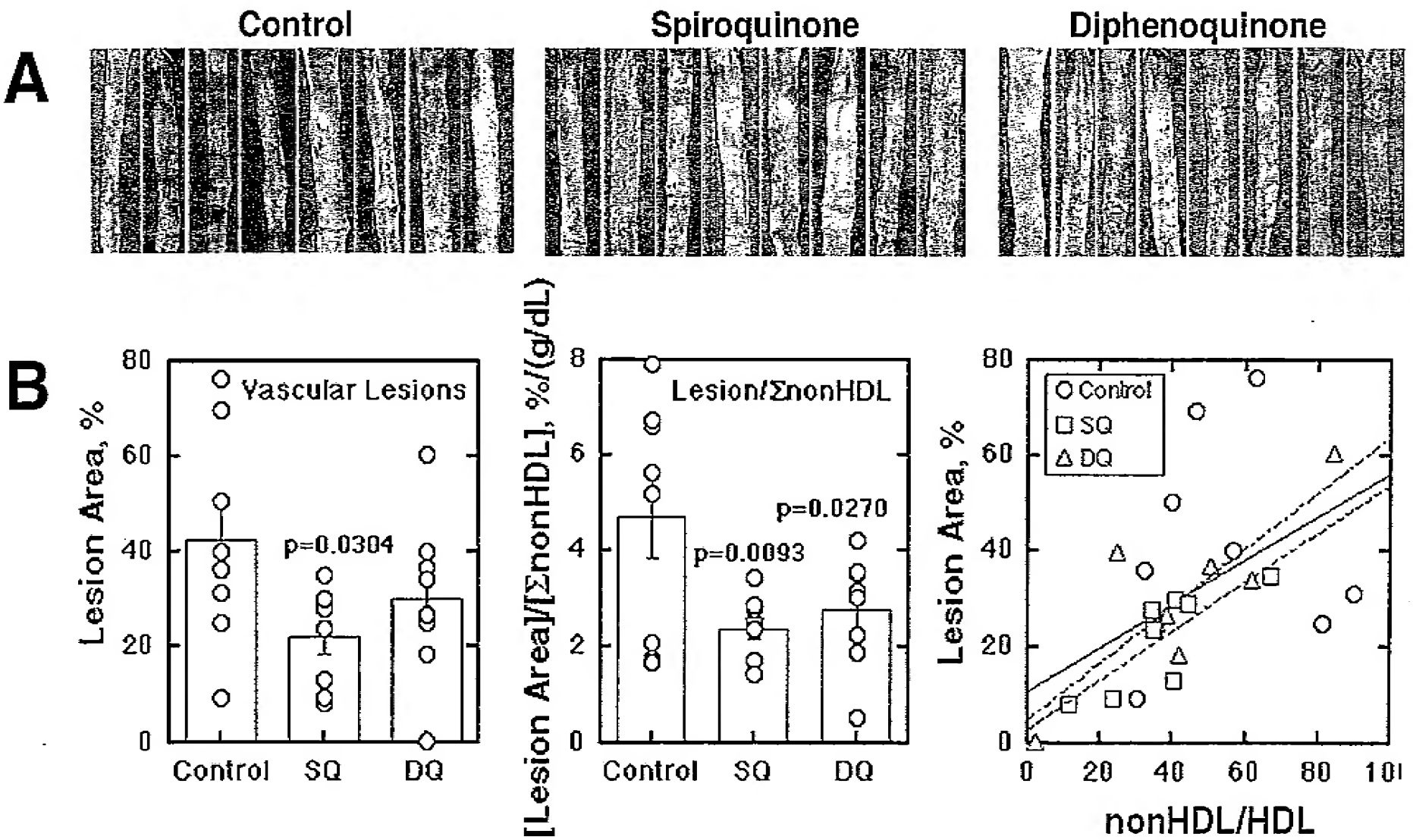
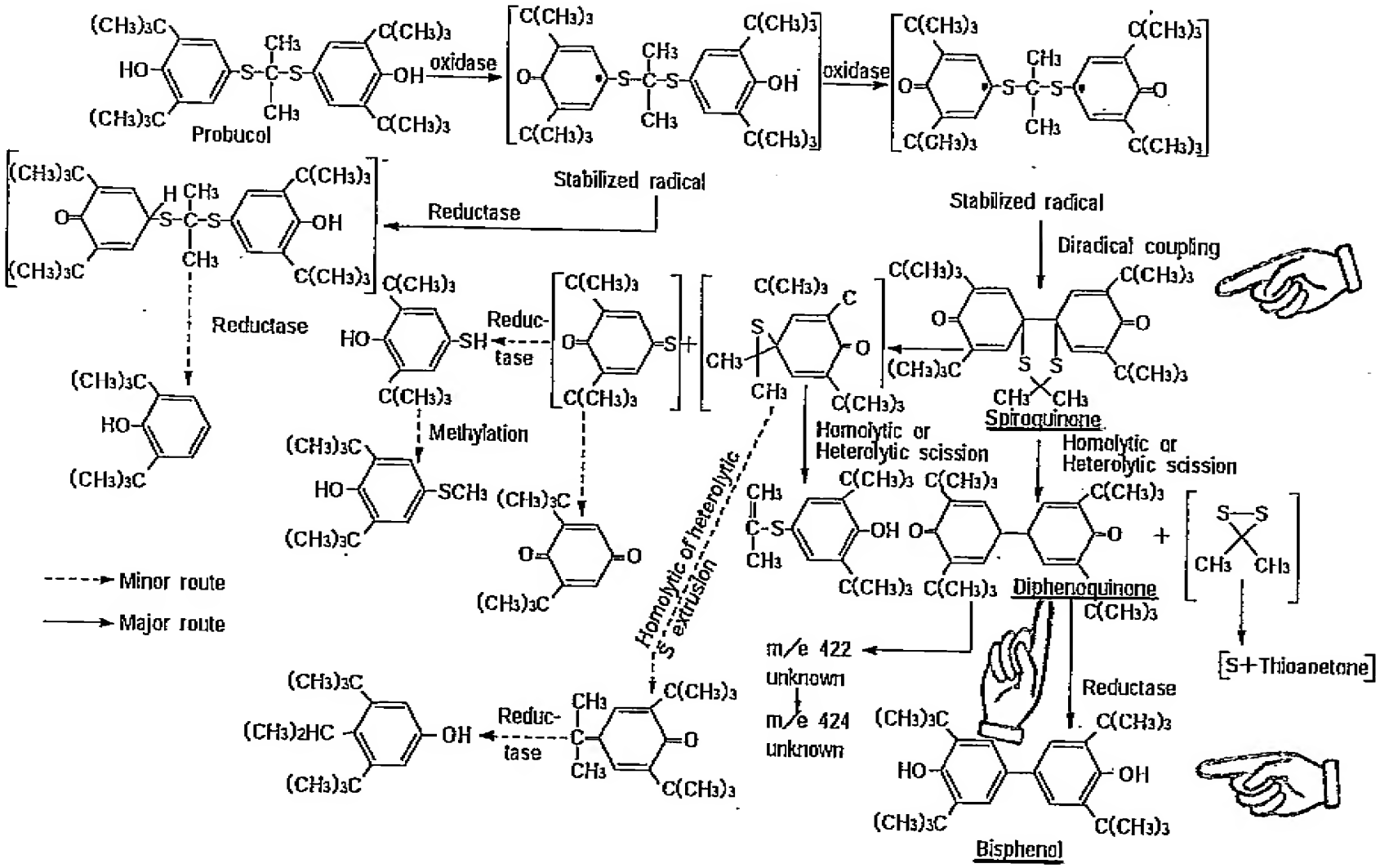


Figure 5, Arakawa et al.



APPENDIX 4



Proposed metabolism pathway of probucol

プロブコールの推定代謝経路